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(54) Title: ISOLATION GROWTH AND DIFFERENTIATION OF HUMAN MUSCLE CELLS

(57) Abstract

Myoblasts are produced in low or serum free medium for use in introduction of mammalian host, particularly human host, for treatment of diseases of muscle tissue or acting as carriers for genetic capabilities, particularly the production of soluble protein products which may serve in the therapy of the mammalian host.

ISOLATION GROWTH AND DIFFERENTIATION OF HUMAN MUSCLE CELLS

INTRODUCTION

Technical Field

The field of this invention is the development of myoblasts for use in the treatment of neuromuscular disease and for transformation for cellular therapy of diseases of diverse etiology.

Background

Myoblasts are precursor cells of the mesoderm that are destined for myogenesis. The determined myoblasts are capable of recognizing and spontaneously fusing with other myoblasts leading to the production of a differentiated myotube. The multinucleated myotube no longer divides or synthesizes DNA but produces muscle proteins in large quantity. These include constituents of the contractile apparatus and specialized cell-surface components essential to neuromuscular transmission. Eventually, the differentiated muscle cell exhibits characteristic striations and rhythmic contractions. A further step in this pathway is maturation; the contractile apparatus and muscle at different stages of development contain distinct isoforms of muscle proteins such as myosin and actin, encoded by different members of multigene families.

Methods have been developed for production of myoblasts from fetal and adult tissue. The success of these methods suggests that it is possible to generate large volumes of myoblasts from adult muscle tissue

that are substantially free of other cells. The myoblasts have the potential for being used in a variety of ways. First, the myoblasts may serve for the treatment of various diseases associated with genetic defects involving muscle tissue. The myoblasts may also be found to be useful as vehicles for cell-therapy, where one or more genes may be introduced into the myoblasts to provide a product of interest.

Relevant Literature

Blau and Webster, Proc. Natl. Acad. USA (1981) 78:5623-5627 describe isolation and cloning of muscle cells for proliferation or differentiation of individual clones. Blau, et al., Proc. Natl. Acad. USA (1983) 80:4856-4860 describe a defect in the proliferative capacity of myoblasts (satellite cells), mononucleated precursors of mature muscle fibers, in clonal analyses of cells cultured from Duchenne muscular dystrophy patients. Blau, et al., Exp. Cell. Res. (1983) 144:495-503 describe the production and analysis of pure myoblast clones from biopsies of patients with Duchenne muscular dystrophy. Blau, et al., Science (1985) 230:758-766 describe the fusion of muscle cells with non-muscle cells with activation of muscle gene expression in the non-muscle cell type. Webster, et al., Exp. Cell Res. (1988) 174:252-265 describe the purification of human myoblasts using a fluorescence-activated cell sorter. Ham, et al., In Vitro Cell. & Dev. Biology (1988) 24:833-844, describe a serum-free medium for clonal growth of human muscle satellite cells. Webster, et al., Cell (1988) 52:503-513, describe that Duchenne muscular dystrophy selectively affects a subset of skeletal muscle fibers specialized for fast contraction (see also the references cited therein).

SUMMARY OF THE INVENTION

Myoblast cells are produced in serum free or low serum media for use in cell-therapy. The myoblasts are capable of migrating, fusing into pre-existing fibers, and may serve as carriers for genes introduced as a result of transformation. The migration of myoblasts across basal lamina allows for a reduced number of injections for treatment of a variety of diseases.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and cells are provided for use in cellular therapies for the treatment of diseases. Methods are described for preparing clonally pure or substantially enriched myoblasts in large amounts in the absence or substantial absence of serum in the nutrient medium. The resulting cells may be used for treatment of a variety of diseases associated with muscle tissue, or other tissue where a soluble factor is involved.

The cells which are employed are myoblasts which may be obtained from tissue samples, which may include fetuses, neonates or tissue from older humans. These cells may be fresh or may be immediately frozen after being taken from the patient, or may be clonal cultures which are grown up from about 5 to 30 population doublings, and then stored frozen for use. The cells are grown in the subject media in a cell culture incubator (37°C, 5% CO2 in air, saturated humidity), as the optimum conditions. Other conditions may be employed, if desired. The chosen medium provides for proliferation, without significant differentiation. Thus, the medium retains the myoblast l vel of maturation and, when desir d, the myoblasts may be introduced into an environment, where they will differentiate and mature.

The medium comprises a source of the essential

amino acids, inorganic salts, trace elements and vitamins, as well as other organic components. The following table indicates what has generally been found to be optimum, although as is known in the field, various changes may be made to individual components without deleteriously affecting the growth of the myoblasts.

TABLE

	Preferred Concentration M/l	Broad Concentration Range M/l
BULK INORGANIC SALTS		
$CaCl_2 \cdot 2H_2O$	1.60×10^{-3}	$10^{-2} - 10^{-4}$
KCl	4.00×10^{-3}	$10^{-3} - 10^{-4}$
$MgSO_4 \cdot 7H_2O$	1.00×10^{-3}	$10^{-2} - 10^{-4}$
NaCl	1.10×10^{-1}	$0.05 - 0.5$
$Na_2HPO_4 \cdot 7H_2O$	5.00×10^{-4}	$10^{-3} - 10^{-4}$
TRACE ELEMENTS		
$CUSO_4 \cdot 5H_2O$	1.00×10^{-8}	$10^{-7} - 10^{-9}$
$FeSO_4 \cdot 7H_2O$	3.00×10^{-6}	$10^{-5} - 10^{-6}$
H_2SeO_3	3.00×10^{-8}	$10^{-7} - 10^{-8}$
$MnSO_4 \cdot 5H_2O$	1.00×10^{-9}	$10^{-8} - 10^{-10}$
$Na_2SiO_3 \cdot 9H_2O$	1.00×10^{-5}	$10^{-4} - 10^{-5}$
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	3.00×10^{-9}	$10^{-8} - 10^{-9}$
NH_4VO_3	5.00×10^{-9}	$10^{-8} - 10^{-9}$
$NiCl_2 \cdot 6H_2O$	3.00×10^{-7}	$10^{-6} - 10^{-7}$
$ZnSO_4 \cdot 7H_2O$	3.00×10^{-7}	$10^{-6} - 10^{-7}$
BUFFERS, INDICATORS AND MISCELLANEOUS		
Phenol Red (Na salt)	3.3×10^{-6}	$10^{-5} - 10^{-6}$
$NaHCO_3$	1.4×10^{-2}	$5x10^{-2} - 5x10^{-3}$

VITAMINS		
d-Biotin	3.00×10^{-8}	$10^{-7} - 10^{-8}$
Folinic Acid (Ca salt)•$5H_2O$	1.00×10^{-6}	$5x10^{-6} - 5x10^{-7}$
DL-alpha-Lipoic Acid	1.00×10^{-8}	$5x10^{-8} - 5x10^{-9}$
Niacinamide	5.00×10^{-5}	$10^{-4} - 10^{-5}$
D-Pantothenic Acid (Hemi-Ca salt)	1.00×10^{-4}	$5x10^{-4} - 5x10^{-5}$
Pyridoxine HCl	1.00×10^{-5}	$5x10^{-5} - 5x10^{-6}$
Riboflavin	1.00×10^{-8}	$5x10^{-8} - 5x10^{-9}$
Thiamin HCl	1.00×10^{-5}	$5x10^{-5} - 5x10^{-6}$
Vitamin B12	1.00×10^{-8}	$5x10^{-8} - 5x10^{-9}$
OTHER ORGANIC COMPONENTS		
Adenine	1.00×10^{-6}	$5x10^{-6} - 5x10^{-7}$
Choline Chloride	1.00×10^{-4}	$5x10^{-4} - 5x10^{-5}$
D-Glucose	5.55×10^{-3}	$10^{-2} - 10^{-3}$
myo-Inositol	1.00×10^{-4}	$5x10^{-4} - 5x10^{-5}$
Putrescine• 2HCl	1.00×10^{-9}	$5x10^{-9} - 5x10^{-10}$
Sodium Pyruvate	1.00×10^{-3}	$5x10^{-3} - 5x10^{-4}$
Thymidine	1.00×10^{-7}	$5x10^{-7} - 5x10^{-8}$

In addition to the basic medium which will be referred to as MCDB120 additional factors are supplied. The first factor is dexamethasone in an amount of 0.3 to 0.5 µg/ml, preferably about 0.39 to 0.40 µg/ml. Serum albumin, particularly bovine serum albumin, will be employed in from 0.25 to 0.75 mg/ml, preferably about 0.50 mg/ml. Epidermal growth factor is employed in from about 5 to 15 ng, preferably about 10 ng/ml. Fetuin is employed in from about 0.25 to 0.75 mg/ml, preferably 0.5 mg/ml. Finally, insulin, conveniently bovine insulin, may be employed in from about 150 to 200 µg/ml, preferably about 180 µg/ml.

To grow the myoblasts, an inoculum is introduced into the medium described above and the cells grown under the conditions described. After adding the inoculum, mild agitation is employed to ensure uniform distribution of the cells for further growth toward confluency.

Cells may be harvested in any convenient way. Tissue may be dissociated for a total of 40-60 min by two or three successive treatments with 0.05% trypsin - EDTA at 37°C in a Wheatin graduated trypsinzation flask with constant stirring. The cells collected in the supernatant after each trypsin treatment are pooled and cooled to 4°C on ice. Horse serum is added to a final concentration of 10% (vol/vol) to terminate further protease activity. The dissociated cells are then centrifuged (2 min, 25°C); the cell pellet is resuspended in conditioned media and either plated in culture or frozen in liquid nitrogen at a density of about 0.1 cm^3 of tissue per ml.

In culture, the myoblasts may be transformed in any of a wide variety of ways, including fusion, transfection, infection, electroporation, ballistics or the like. The particular method for introducing the foreign DNA is not crucial to this invention. Depending on the purpose for the introduction of the DNA, there may be an interest in having directed homologous or legitimate integration by recombination or illegitimate recombination. See Smithies, et al., (1985) Nature 317:230-235; Thomas and Capecchi (1987) Cell 51:503-512 and Mansour, et al. (1988) Nature 336:348-352. For directed integration, the gene of interest will be flanked by at least 50 bp on each side of DNA homologous to the site for integration, usually at least 100 bp, and the total of homologous DNA may be as high as 10 kbp, usually not greater than about 5 kbp, where preferably the flanking regions will be of about the same size.

Regions for integration may include DNA sequences associated with a particular muscular defect. Thus, the host myoblasts may be removed from the host, transformed by homologous recombination, and cells cloned and screened for homologous recombination at the site of the defect. Alternatively, where a naturally occurring inducible gene is involved, which is normally supressed in a myoblast or mature muscle tissue, one may provide for homologous recombination, where the transcriptional initiation regulatory sequence, e.g., promoter with or without an enhancer, is modified to provide for a different basis for induction or for constitutive transcription. Thus, the myoblasts may then be used for expression of an endogenous gene (native to the host) or heterologous, which is normally not expressed in muscle tissue. For example, one may wish to provide for expression of cytokines, growth factors, colony stimulating factors, interferons, surface membrane receptors, insulin or the like. By modifying the transcriptional initiation regulatory region, the myoblasts may provide for constitutive production of the expression product or alternatively or in combination, one may introduce a receptor for the soluble product, which provides for inducible transcription of a cellular, e.g., cytoplasmic, nuclear, etc., protein. By activating the receptor, the myoblasts may be induced to produce the expression product under the induction of the relevant ligand.

Various vehicles or vector constructs may be employed for the transformation of the myoblast cells. Of particular interest for transfection or infection are replication-defective viral vectors, DNA virus or retroviral vectors, which may be introduced into the cells. The vectors will normally be free of any prokaryotic DNA and may comprise a number of different functional sequences. As already discussed,

one of the functional sequences may be a DNA region comprising transcriptional and translational initiation and termination regulatory sequences, an open reading frame encoding the protein of interest, and may further comprise flanking regions for site directed integration. In some situations, as already indicated, the 5'-flanking region will provide for homologous recombination to change the nature of the transcriptional initiation region. For example, the presence or absence of an enhancer may be modified, to provide for inducible transcription or non-inducible transcription, to increase or decrease the level of transcription, or the like. Similarly, the promoter region may be modified, so as to be more or less susceptible to induction, to increase or decrease the level of transcription, or the like.

The structural gene which is employed may result in an intracellular product, i.e., retained in the cell, in the cytoplasm or organelle, e.g., the nucleus, in transport to a membrane, either an intracellular membrane or the cell membrane, or for secretion by providing for the natural signal sequence present with the structural gene or a signal sequence which is not naturally present with the structural gene. In some situations, where the soluble protein of interest is a fragment of a larger protein, it may be necessary to provide a signal sequence with such protein, so that upon secretion and processing at the processing site, the desired protein will have the natural sequence.

A marker may be present for selection of cells which contain the vehicle construct. Normally, the marker will allow for positive selection, in providing protection from one or more cytotoxic agents. For example, kanamycin resistance may be empl yed, where the cells may be selected with G418, dihydrofolate reductase may be employed for resistance to

m thotrexate, and the like. The marker may be an inducible or non-inducible gene, so that selection may occur under induction or without induction.

The vector may also include a replication origin and such other genes which are necessary for replication in the host. The replication system comprising the origin and any proteins associated with replication encoded by the particular virus may be included as part of a construct. Care must be taken in selecting the replication system, so that the genes which are encoded for replication do not provide for transformation of the myoblasts. Illustrative replication systems include Epstein-Barr virus. Alternatively, replication defective vehicles may be employed, particularly replication-defective retroviral vectors. These vectors are described by Price, et al., Proc. Natl. Acad. Sci.(1987) 84:156-160 and Sares, et al. EMBO J. (1986) 5:3133-3142. The final vehicle construct may have one or more genes of interest. Either a cDNA gene or a chromosomal gene may be employed. Of particular interest is to provide for at least one intron, which may be present in the 5'-non-coding region or in the coding region. It is found that the presence of an intron enhances stability of the messenger RNA.

Alternatively, cells may be transformed in vivo by injection of replication-defective viral vectors, which are infectious. The vectors may be introduced into retroviral producer cells for ecotropic packaging. The cells are then collected, filtered and concentrated by centrifugation and the viral stock may then be injected into a site in vivo. Since it is found that the myoblasts will migrate, relatively few injections into the muscle fibers are required, since the myoblasts will expand into adjacent regions.

Administration of the vector is conveniently by injection in a physiologically acceptable medium,

such as water, saline, phosphate buffered saline, or the like. The viral concentration will generally be from about 10^5 ffu. Other additives which may be present include polybrene. Usually, the injections will be about 10^5 cells per cm^3 of muscle tissue. The trauma to the tissue may be substantially minimized by having only a few injections in the region of interest. Particularly, where a patient may have need for extensive treatment, the desirability of having a low number of injections in a particular area is manifest.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Vectors

Two ß-galactosidase vectors were employed, referred to as BAG and pMMuLVSVnlsLacZ. The two vectors each contain ß-galactosidase encoding sequences under the transcriptional control of the MMuLV promoter/enhancer or the SV40 early promoter. The vectors are further characterized by neo^r and LacZ genes, and in some cases 7 amino acid codons for the SV40 large T nuclear localization sequences.

Supernatant from CREBAG2 or PA12$\Psi_2$12-C2 retroviral producer cells made by transfecting BAG into the Ψ_{CRE} or pMMuLVSVnlsLacZ into the Ψ_2 ecotropic packaging cell lines that are recombination resistant (Price, et al., 1987; Sanes, et al., 1986), respectively was collected, filtered and concentrated by centrifugation. Viral stock (50-200 µl) was mixed with charcoal particles and 10 µM polybrene and injected from a 26 gauge needle into the latero-dorsal surface of anaesthetized Wistar rat hindlimbs. Animals were allowed to develop for about two weeks, then fixed by cardiac perfusion with 4% paraformaldehyde, 0.5%

glutaraldehyde, 100 mM PIPES pH 7.4.

After 30-60 min lower hindlimbs were dissected free of skin and placed in the same fixative overnight at 4°C, then in 30% sucrose phosphate buffered saline (PBS) for 24 h at 4°C, frozen in freezing isopentane and cut into serial 30 µm sections on a cryostat. Sections were post-fixed in 2% paraformaldehyde, washed and stained in 1 mg/ml X-gal, 35 mM potassium ferri- and ferrocyanide, 1 mM $MgCl_2$ in PBS overnight at 30°C, mounted in glycerol:PBS (9:1) and examined under bright field optics with a Zeiss Axiophot microscope for the presence of the blue X-gal reaction product. Clusters of muscle fibers stained blue were scattered throughout the lower hind limb. Charcoal particles were generally located between soleus and lateral gastrocnemius and were used to identify the site of infection. No differences in the distribution or size of clusters of labeled muscle cells was observed with either the BAG or pMMuLVSVnlsLacZ vectors. The BAG vector was injected at P9 (P = postnatal) and analyzed at P23 and the pMMuLVSVnlsLacZ was injected at P16 and analyzed at P29. In addition, clusters of labeled cells close to the charcoal particles were not detectably different from those several millimeters away, suggesting that the injection did not perturb development of nearby tissue.

It was found that with rats injected at 9 days and analyzed at 23 days, the number of clusters spanning multiple fibers was 16 of 25, while with rats of 19 days analyzed at 35 days, the number of clusters spanning was 1 of 1.

Clusters of stained muscle fibers in the lateral gastrocnemius muscle demonstrated that the myoblasts could be infected and expressed β-galactosidase even after fusion into multinucleate fibers. Many of the clusters observed represented clones derived from single cells, some of the progeny of which

migrated across basal lamina of a given muscle fiber and fused into adjacent muscle fibers. Since each myoblast is associated with a single fiber at the time of infection, the data indicate that myoblasts are capable of migrating through the basal lamina from one fiber to another. Moreover, since the majority of infection events yielded clones spanning multiple muscle fibers, migration appears to be a relatively frequent event.

To demonstrate that each cluster of β-galactosidase-positive cells in an infected rat leg originated clonally from a single retrovirally infected myoblast, a mixture of two vectors were injected that generate distinct β-galactosidase staining patterns: nuclear and cytoplasmic. To maximize the number and size of clones, a mixture of the two vectors was injected into P0 rat hindlimbs, a time when extensive proliferation and fiber formation is occurring. In two legs heavily infected with 87 separate β-galactosidase-positive clusters of which 23 contained cytoplasmic β-galactosidase, only in two instances did fibers adjacent to a cytoplasmically-stained fiber contain nuclear staining. Thus, the frequency with which adjacent myoblasts are infected independently is below 15% in animals infected at a level of 40-50 clones per lower hindlimb. Hence, in the less heavily infected hindlimbs, it is unlikely that more than one or two of the clones are in fact derived from two or more separate infections. The vast majority are clones derived from a single infection event.

Employing the dual virus technique, whether each labelled fiber arose from a separate infection event was investigated by determining whether there was a random distribution of cytoplasmic and nuclear stains among all fibers labelled. The contrary was observed, in that cytoplasmic and nuclear staining patterns were segregated into distinct areas, each including a

cluster of several fibers.

It is unlikely that new fibers formed during the period of the investigation, since the number of fibers apparently remained constant. The mean diameter of fibers within clones was similar to the overall mean fiber diameter for the muscle suggesting no preferential inclusion of small, newly-formed fibers within multifiber clones. The high frequency of multifiber clones compared with the maximum possible rate of new fiber formation suggest that the myoblasts destined to form new fibers would have had to have been highly preferred for infection by the vector compared to the bulk population of dividing myoblasts. The data strongly support the migration of myoblasts through the basal lamina as the mechanism by which transformed myoblasts are included in different fibers.

It is evident from the above results that the subject invention allows for the use of muscle forming cells in the treatment of diseases associated with muscle tissue or for production of soluble or other proteins in a host. The myoblasts are capable of forming new muscle tissue or participating in the formation of fibers, where the cells may provide for useful properties, correct defects, and the like. In addition, the cells may be modified with markers, to allow for selective advantage of the transformed cells over the naturally present cells.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. Mammalian muscle tissue in a mammalian host comprising myoblasts or progeny thereof, wherein said myoblasts were grown in culture and are present as a result of introduction of said myoblasts from said culture into said muscle tissue.

2. Mammalian muscle tissue according to Claim 1, wherein said myoblasts or progeny thereof are introduced at one or more sites of said tissue, and said myoblasts or progeny thereof are present at sites distal from said site of introduction.

3. Mammalian muscle tissue according to Claim 1, wherein said cells are grown in culture in at least substantially serum-free culture medium.

4. Mammalian muscle tissue according to Claim 1, wherein said at least substantially serum-free medium comprises a sufficient amount of dexamethasone, serum albumin, epidermal growth factor, fetuin and insulin to provide for growth of said myoblasts without differentiation.

5. Mammalian muscle tissue according to Claim 4, wherein said myoblasts comprise a DNA construct as a result of in vitro introduction of said DNA construct in said myoblast.

6. Mammalian muscle tissue according to Claim 1, wherein said myoblasts comprise a DNA construct as a result of in vitro introduction of said DNA construct into said myoblasts.

7. Mammalian muscle tissue according to Claim 6, wherein said construct comprises a gene capable of

expression in said myoblasts.

8. Myoblasts or myotubes comprising a DNA construct as a result of synthesis of said construct in vitro and transformation into said myoblasts, wherein said myoblasts proliferate and form myotubes.

9. Myoblasts or myotubes according to Claim 8, wherein said transformation is by transfection.

10. Myoblasts or myotubes according to Claim 8, wherein said transformation is by infection.

11. Myoblasts or myotubes according to Claim 10, wherein said infection is with a replication-defective retrovirus.

12. Myoblasts or myotubes according to Claim 8, expressing a product encoded by said gene.

13. Myoblasts according to Claim 8, wherein said myoblasts are adult myoblasts.

14. A method for modifying the genome with a DNA construct of interest capable of transcription of myoblasts and myotubes in muscle tissue, said method comprising:

contacting said muscle tissue with a replication defective virus comprising said DNA construct, whereby said virus transforms said myoblasts with said DNA construct.

15. A method according to Claim 14, wherein said virus is a retrovirus.

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